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(54) Title: TOTAL SYNTHESIS AND FUNCTIONAL OVEREXPRESSION OF A *CANDIDA RUGOSA* LIP1 GENE CODING FOR A MAJOR INDUSTRIAL LIPASE

(57) Abstract

The dimorphic yeast ***Candida rugosa*** has an unusual codon-usage which hampers the functional expression of genes derived from this yeast in a conventional heterologous host. Lipases produced by this yeast are extensively used in industrial bioconversions, but commercial lipase samples contain several different isoforms encoded by the *LIP* genes family. In a first laborious attempt the *LIP1* gene, encoding the major isoform of the *C. rugosa* lipases (CRLs), was systematically modified by site-directed mutagenesis to gain functional expression in *S. cerevisiae*. Asalternative approach, the gene (1688 bp) was completely synthesised with an optimised nucleotide sequence in terms of heterologous expression in yeast and simplified genetic manipulation. The synthetic gene was functionally overexpressed in *Pichia pastoris*. The recombinant CRL was produced at high level and purity, accounting for 90-95 % of the secreted proteins. The physical-chemical and catalytic properties of the recombinant lipase were compared with those of a commercial, non-recombinant *C. rugosa* lipase preparation.

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Total synthesis and functional overexpression of a *Candida rugosa LIP1* gene coding for a major industrial lipase

INTRODUCTION

Lipases (triacylglycerol lipase EC 3.1.1.3) catalyse the hydrolysis of triglycerides at water/oil interfaces. In vitro, however, they are versatile enzymes because of their ability to catalyse the hydrolysis and synthesis of a great variety of esters (i.e. the hydrolysis and transesterification of triacylglycerols), or the resolution of racemic mixtures. They find applications also as industrial detergent (i.e. due to their ability to remove fat and ink stains and in food industry where they are used for fat retailoring of triglycerides.

Candida rugosa lipases (CRLs) are among the commercial lipases most often employed in hydrolysis and synthesis of a wide range of esters of commercial interest [1]. In most biocatalytic applications crude enzyme preparations, obtained by TCA precipitation of the culture supernatant, are applied [2].

In agreement with the presence of several lipase genes in *Candida rugosa* [3,4], several lipase isoforms have been isolated from commercial enzyme preparations, where the gene product of *LIP1* constitutes the major lipase form [5,6]. The expression of different genes coding for distinct isoforms seems to be regulated by the cultivation conditions (data not published). Notwithstanding the high homology among the cloned genes (60-70% sequence identity), differences in glycosylation of these lipases may also contribute to their heterogeneity [6].

Since the isoforms might differ in their catalytic performances[6] and some purification procedures affect the conformation of the lipases[7,8], the cloning and the separate expression of lipase genes seems to be the most suitable approach for the production and characterisation of pure isoforms and will also allow the production of mutant lipases with optimised properties for biocatalytic applications.

However, *Candida rugosa* is a dimorphic yeast in which, as in some other phylogenetically related *Candida* species [9], the triplet CUG, a universal codon for leucine, is read as serine [10]. This unusual amino acid assignment of the CUG codon relies on the presence in *C. rugosa* of an unusual $tRNA^{Ser}$ with an anticodon CAG [11]. In most *Candida* species, CUG is

extremely rare, with the notable exception of *Candida rugosa* where it accounts for about 40% of the total serine codons [3,4]. Accordingly, multiple copies of the genes coding for $tRNA^{Ser}CAG$ have been isolated from the genome of this yeast [11].

As a consequence of this unusual codon-usage, the heterologous expression of *LIP1* in *S.cerevisiae* resulted in an inactive lipase [12]. Hence the exchange of the CUG by universal serine triplets is required for the expression of a functional protein in heterologous hosts. This has been carried out previously for the heterologous expression of two other genes encoding cytochrome P450 cloned from another *Candida* microorganism i.e. *C. maltosa* [9]. The expression of these genes in *S.cerevisiae* however resulted in the formation of still active but unstable enzymes and thus cannot be considered a succesful route for expression of industrially useful expression products. The occurrence of this affected phenotype is postulated to be related to the low frequency of CTG codon in these genes. In general, the CTG codon has a rather low frequency in the *C.maltosa* genome and has only been found so far in *P450alk* genes, *HIS5* and *URA3* among the *C.maltosa* genes sequenced.

On the contrary, in *Candida rugosa* lipases, CTG is used with a high frequency (3% of the codons), including that corresponding to the catalytic Ser. 209 In the *LIP1* gene (ORF 1647 nt), 20 out of 42 serine residues are encoded by CUG triplets [3]. There are 20 CUG serines: 19 in the mature protein and 1 in the leader sequence.

Alberghina and Lotti (1997)[26] describe an attempt to express LIP1 in *S. cerevisiae* and faced unexpected issues concerning the translatability/stability of the recombinant protein in host cells and the non universal genetic code utilized by *Candida* cells. Insertion of the lipase gene in *S.cerevisiae* resulted in abundant mRNA production as witnessed by Northern blot but no trace of recombinant protein in either cells or medium. Replacement of the first 15 amino acids of lip 1 with another leader sequence i.e. that of killer toxin from *Kluyveromyces lactis* gave mRNA and intracellular accumulation. This is also disclosed in reference 12 which also states it was inactive. They describe mutation of codons encoding conserved serines 209, 241 and 282 by PCR. The resulting expression product was accumulated intracellularly in an inactive form. A second group was taken into account due to their structural role according to the hydrogen bonds and salt bridges and also mutagenised also to no avail. In summary the expression of active *C. rugosa* in a heterologous host did not seem feasible. No active form was

available, no secretion occurred.

Our first attempt towards the expression of functional CRL proteins comprised more extensive mutagenesis of a natural gene coding for lipase (*LIP1*) and its expression in *S.cerevisiae*. We tried to overcome the problems occurring in the heterologous expression of the natural *LIP1* gene by site-directed mutagenesis in order to replace the CTG codons by other universal codons for serine. Since it would be a tremendous task to change all 19 CTG codons, we tried to define a hierarchy of the functionally and structurally most important Ser residues to be mutated.

Based on the alignment with other Ser hydrolases and on the 3D structure of the enzyme we selected a group of 8 serines as the target for the mutagenesis. Our selection resided in the fact that these residues, are either highly conserved within the proteins belonging to the lipases/esterases family [18] or may be of structural importance.

A series of mutant genes, differing with regard to number of restored serine residues (2, 3, 5, 8) was produced (See tab. I).

Since it was previously demonstrated that the natural *LIP1* is efficiently synthesised by *S.cerevisiae* cells only following the replacement of the endogenous leader sequence with the signal peptide of the *Kluyveromyces lactis* killer toxin [12,19] i.e. apparently because the *S. cerevisiae* does not process the native *C. rugosa* lipase presequence, we constructed a hybrid form for the mutant lipase genes, analogously to what was reported by the same authors. The hybrid lipase mutant genes were cloned into pEMBLyex4, a shuttle expression vector for *S.cerevisiae* cells containing the inducible *UAS GAL* sequence [20]. Recombinant yeast cells grown under inducing conditions, produced high levels of protein, but the resulting recombinant lipase was found to accumulate intracellularly at about 10-20 mg/l culture in a non active form. The same result was obtained for all 4 mutants, independently of the number of Ser residues restored.

Although the protein glycosylation provided indications in favour of the correct targeting of the chimeric mutated protein to the endoplasmatic reticulum, its secretion failed, suggesting difficulties at the posttranscriptional level, which might be connected with the mutations (13/19) still present in the molecule. These mutations could affect the folding of the protein, hampering in turn, its correct processing through the secretory pathway. It is also possible the signal sequence is relevant for activity thus explaining inactivity upon

replacement of the signal sequence by a non native presequence. If this is the case then expression in a heterogenous host cell of an active *C. rugosa* lipase would be impossible. Production would then only be possible using a *C. rugosa* host cell with concomitant contamination with other lipases of *C. rugosa* of which as indicated above a number are known (see e.g refs.[3] and [4]. In addition the industrial process of protein production with the host *C. rugosa* is not as well examined and optimised as for other yeasts such as *S. cerevisiae* with the obvious concomitant disadvantages for industrial use. It did not seem likely the mutagenesis of further serine codons would have an effect as the most relevant candidates vital to activity had already been mutagenised. In addition the task of undertaking such large scale mutagenesis is tremendous i.e. an awful lot of effort with very little expectation of success.

The subject invention comprises the chemical-enzymatic synthesis of a gene coding for a natural *C. rugosa* lipase and the expression thereof in a heterologous host cell in an active form, moreover the secretion thereof to such a degree that a supernatant comprising lipase with contamination of less than 20% with other protein is obtained. As no other protein is visualised by SDS-PAGE and silver staining we can actually achieve purity higher than 90%. The synthetic gene synthesis represents one of the most ambitious gene syntheses to date. Any commercial preparations known in the art comprise active lipase from *C. rugosa* in numerous isoforms, other proteins and stabilisers. In general purity levels are lower than 80%, thus the instant invention offers a major step forward in providing substantially purer *C. rugosa* lipase. The *C. rugosa* lipase according to the invention will be free of other *C. rugosa* lipase isoforms. It is now possible to produce *C. rugosa* lipase I e.g. free of lipase II-V on an industrial scale.

The subsequent recombinant gene was introduced into and expressed in a heterogenous yeast. In a preferred embodiment we used *P. pastoris*, a methylotrophic yeast that has become increasingly attractive as host in the production of heterologous proteins [13]. The system has been well developed for industrial-scale fermentations and *Pichia pastoris* can be grown to high cell density in relatively inexpensive media. No stable multicopy vectors exist for *P.pastoris* and foreign genes are integrated into the genome for expression. The secretion by *Pichia* cells requires the presence of a signal sequence fused to the expressed protein. Several different secretion signals, including the native secretion signals present on some heterologous proteins [14], have been found to be effective

previously in *P. pastoris*.

The secretion signal sequence from the *S.cerevisiae* α-factor prepro peptide forms a preferred embodiment in a gene construct according to the invention. This presequence has been used with success previously in other situations[15]. In an alternative embodiment the expression of the synthetic lipase gene was carried out in S. cerevisiae. *Hansenula* is also a host cell which is attractive for commercial production purposes.

In addition physical-chemical and catalytic properties of the recombinant lipase were compared with those of commercial CRLs. Surprisingly we found that high level expression of active and pure recombinant lipase occurred. In fact this occurred to such a degree as to make its industrial production economically feasible. Thus, a long existing problem, i.e. industrial production of sufficiently pure and active *Candida rugosa* lipase has now been solved.

DETAILED DESCRIPTION OF THE INVENTION

Site-Directed Mutagenesis on *C.rugosa* lipase gene

Five members belonging to the multigene family coding for lipase in *Candida rugosa* have previously been cloned and sequenced [16]. The present invention was carried out using the *LIP1* sequence. This sequence codes for the major lipase isoform, the best characterised isoform for which the crystallographic structure has been solved[17]. The genomic sequence of *LIP1* contains a unique ORF of 1647 nt terminating with a TAA stop codon. The deduced aa sequence corresponds to a protein of 549 aa with an N-terminal stretch of 15 hydrophobic aa, encoding a signal peptide. The LIP1 mature protein contains 42 Ser residues, 19 of them encoded by CUG triplets [3].

Construction of a synthetic, codon-optimised *C.rugosa* lipase gene

In the approach according to the invention to achieve functional expression of *LIP1* in conventional yeasts, a sequence identical to the native *LIP1* wherein all CTGs have been replaced by universal Ser codons was completely synthesised by the polymerase chain reaction technique, according to the method of mutually priming long overlapping oligonucleotides[21]. This was in itself a major undertaking in which numerous problems were encountered.

The nucleotide sequence of the *LIP1* gene was modified in accordance with a synthesis strategy that provides for the subdivision of the gene into 4 segments of ca. 400 bp which are separately synthesised (Fig. 1). In first instance the resulting segments did not result in a correct active lipase. The use of relatively long oligo primers was unsuccesful and major deletions and missequences apparently occurred. A second attempt using shorter primers resulted in improved sequences with a lot lower frequency of mistakes. A repair strategy was subsequently developed and caried out to ensure a mistake free sequence was generated. In an alternative embodiment the codon usage of *S.cerevisiae* was taken into account. Naturally the codon usage can be adapted to suit the heterologous host of choice. We found the *S. cerevisiae* adapted sequence worked well in *P. pastoris*. Thus a nucleic acid sequence in which the C and G nucleotide level has ben reduced below 63% and wherein the amino acid identity remained that of the native protein forms a suitable embodiment of the invention.

A number of changes was thus introduced into the synthetic version, thereby maintaining 100% amino acid identity of the encoding sequence with that encoded by the native mature major lipase encoding gene of *C. rugosa*.

1) All CUG codons were exchanged for universal serine codons.
2) The general C+G content was lowered, according to the DNA composition of the host yeast cell (*C.rugosa* has 63% of C+G).
3) Unique restriction sites were strategically positioned throughout the gene to facilitate both, the analysis of primary subclones containing the four blocks and the construction of the gene by their assembly. With the same purpose, some restriction sites were also removed without changing the coding region.
4) In order to facilitate the expression in *S.cerevisiae* cells, several codons poorly represented in *S.cerevisiae* coding sequences, were exchanged to highly used codons [22]. The final codon-optimised version of the CRL gene of the Example had 77% nucleic acid identity with the natural gene and 100% a.a. identity.

Suitably the nucleic acid sequence according to the invention can be further adapted by removing restriction enzyme sites from the coding sequence that are common to expression vectors in which the gene is to be expressed. The HindIII site is e.g. a site common to numerous expression vectors thus it simplifies handling the sequence if the HindIII recognition sequence present in the coding sequence is removed. It is

preferable to replace such a sequence by nucleotides that maintain the amino acid identity of the non manipulated restriction enzyme recognition site. Another suitable adaptation of the sequence according to the invention lies in the introduction of restriction enzyme recognition sites at points of interest thereby maintaining the original amino acid identity. Preferably any such introduced restriction site will be unique to the sequence, thus if multiple sites are introduced each will be different. A favorable alternative comprises additional estriction enzyme recognition sites at the 5' and 3' termini of the nucleic acid sequence according to the invention thereby enabling introduction of the sequence encoding lipase according to the invention into an expression vector.

It will be apparent to a person skilled in the art that nucleic acid sequences comprising slight variations on the native sequence for each specific lipase can be designed. Such variants fall within the scope of the subject invention. Each native lipase can be characterised by its specific activity. Thus a variant sequence encoding a protein having such specificity is covered by the invention. Such a variant will encode a protein exhibiting at least the degree of activity of the corresponding native protein as expressed from a nucleic acid sequence encoding the native protein. The specificity can be determined analogously to the method illustrated in the examples, e.g. Natural variants in gene sequence may also occur and synthetic nucleic acid sequences encoding such natural variants are also envisaged as falling within the scope of the invention. In summary a recombinant substantially pure lipase as expressed by a heterologous host cell from nucleic acid in which at least 60% of the serine encoding CTG codons have been replaced by universal serine codons aswell as the corresponding nucleic acid sequence and applications thereof are covered by the invention. In particular, the expression product of said sequence should exhibit lipase activity equal to or better than that of the corresponding native lipase. Preferably more than 70% and suitably more than 80% of the CTG codons at serine encoding positions are replaced. To ensure no loss of activity it is preferred to amend as many serine codons as possible, preferably more than 90%. An embodiment where all serine codons have been replaced has been found to function exceedingly well.

The amino acid sequences and nucleic acid sequences of C. rugosa lipases 1-5 are known in the art. It is thus clear what native nucleic acid sequences are for all 5 lipases. The subject invention covers nucleic acid sequences encoding one isoform of C. rugosa lipase either in

its native form or as a variant with the amended serine codons according to the invention as already disclosed. The variant nucleic acid sequence can have a different amino acid sequence than the native lipase but in addition a suitable embodiment should be capable of hybridising to the native sequence under stringent conditions as defined in the art[29]. A further particular embodiment of a variant lipase 1 sequence will exhibit amino acid identity with the known lipase 1 sequence of more than 88%, preferably more than 90%. A suitable embodiment will exhibit 100% identity at amino acid level. Naturally the sequence according to the invention does not only merely have to consist of the mature encoding sequence but it may also comprise a precursor sequence of choice or a part thereof. Preferably such a precursor sequence will be removed during protein production, however it is envisaged that precursor fragments of the mature protein can remain present without affecting activity. Such a remaining preceding sequence should not be longer than 10 amino acids preferably not longer than 5 amino acids in length and should not affect activity or specificity in a negative manner. It is possible to remove such a precursor fragment after isolation of the protein however it is preferable the host cell processes the protein during culture to the mature protein per se for a most cost effective efficient production process. Obviously it is also preferable the host cell secretes the protein in order to facilitate isolation of the protein. Thus a nucleic acid sequence encoding a ripening form i.e. a mature, pre, pro, prepro protein or even a mature protein preceded by a short sequence of less than 10 amino acids is envisaged as falling within the scope of the invention. The 5' terminus of the encoding sequence for the mature protein can be preceded by a leader sequence heterologous to *C. rugosa*. Suitably such a leader sequence is derived from a yeast cell in particular if the sequence is to be expressed in a yeast host cell. A number of suitable sequences are available e.g. from *S. cerevisiae*.

It will be apparent that not only the nucleic acid sequences per se according to the invention are considered to fall within the scope ofthe invention but also an expression vector or a heterologous host cell comprising such a sequence i considered to be covered. in particular when the sequence is operatively linked to a promoter and is thus capable of being expressed. Such a sequence can be single or multiple copy depending on the desired degree of expression. The sequence according to the invention can differ in regard of the host microorganism. It is preferable to optimise codon usage per expression host.

Culturing a microorganism according to the invention can lead to production of lipase to a degree such that said lipase is contaminated at the most by 20% of other protein in the supernatant of a culture of said microorganism. Obviously the most pure composition obtainable can be preferred for particular processes. It has now become possible in an economically feasible manner to achieve contamination with less than 10% and even less than 5% other protein. More specifically one can acnieve *C rugosa* free of other *C. rugosa* lipases. Thus lipase 1 free of 2-5 and lipase 2 free of lipase 1 and 3-5 etc can now be obtained without requiring extensive and expensive working up procedures. In particular a lipase 1 free of 2-5 wherein lipase 1 is defined according to sequence id nr 1 has been illustrated in the Examples. Pure lipase 1 according to the invention has been illustrated as exhibiting higher activity towards caprinate (C10) than towards palmitate (C16) as can be determined by pH stat assay and is illustrated in the examples. A number of characteristics of a lipase 1 according to the invention can be seen in addition in Table II.

As indicated the object of the invention was to provide pure lipase on an industrial scale at a reasonable price. Thus a process for industrial scale production of a lipase according to the invention in any of the embodiments or combinations of embodiments disclosed comprising cultivation of a microorganism according to any of the embodiments disclosed followed optionally by isolation of the resulting expression product in a manner known per se for other protein production processes in industry is also considered to fall within the scope of the invention. Particularly when the lipase is present in the culture supernatant in an amount comprising over 80% of the total *C. rugosa* protein present or even of the heterologous host cell.

With the possibility of achieving such purity of the various lipase isoforms it now becomes possible to harness their specific specificities in particular processes. For lipase 1 for example a process requiring high specificity towards acyl chains shorter than C14 on an industrial scale can be optimised and standardised upon application of the pure product according to the invention. Such improved applications also fall within the scope of the invention.

Expression of recombinant CRL in *Pichia pastoris*

The synthetic gene containing its natural leader sequence, was modified *via* PCR and a hybrid version of the recombinant *LIP1* gene

(*rLIP1*) was obtained by fusion of the nucleotide sequence coding for the mature LIP protein, with the *S.cerevisiae* prepro α-factor peptide (prepro-*rLIP1*).

The subsequent recombinant lipase gene was inserted into the vector pPICZαB generating the plasmid pPICpp*LIP* for the expression in *P.pastoris*.

(1) Expression of C. rugosa *synthetic genes* in P. pastoris

Pichia pastoris is a yeast capable of metabolising methanol as its sole carbon source. The enzyme alcohol oxidase (AOX) is involved in the metabolism of methanol and its transcription is tightly regulated and strongly induced by methanol (more than 30% of the total soluble protein in cells grown with methanol as carbon source). The *rLIP1* was cloned in pPICZαB vector, a shuttle expression vector that contains the *AOX1* promoter as control element of the gene expression [23]. Naturally other vectors can suitably be used. An alternative vector is for example pGAPZ for *P. pastoris*.

Direct evidence of lipase activity was provided using minimal tributyrin-methanol plates, where the positive colonies appeared surrounded by a clear halo as compared to the opaque background of the medium, due to the tributyrin emulsion. A suitable negative control (*Pichia* cells transformed with pPICZαB) never formed halos, even after several days of incubation under inducing conditions.

Selected mutants were further grown in small volume cultures (20ml) in a shaking flask until they reached an OD_{600} of 2-6 and were then transferred to BMMY medium for induction of lipase expression.

After 48 h of induction, lipolytic activity was detected in the supernatant by pNPP assay, showing a significant variability among clones (1 to 20 U/ml lipase). This variability in secretion level could be related to a gene dosage effect, since it has been found that *P. pastoris* strains containing many integrated copies of a foreign gene can yield much higher levels of foreign protein [24]. This phenomenon could rely on the multiple insertion occurring by a mechanism of *in vivo* circularisation of transforming DNA and accounts for a strong difference of expression in shake flasks, also reported by other authors [25]. In a suitable embodiment of the invention the recombinant host cell can thus comprise multiple copies of the nucleic acid according to the invention High producing clones were selected and grown in 2 l Erlenmeyer flasks

containing 200 ml BMGY medium. After 5 days induction, the lipase activity of the supernatants was 85 U/ml.

Culture supernatants were subjected to SDS-PAGE and a unique band at 60 kDa corresponding to the expected molecular weight for CRL was detected (Fig. 2). The same band was visualised after Western blotting analysis using lipase-specific antibodies for immunodetection. Coomassie blue stained gel of the flask culture supernatant allowed visualisation of a protein purity of approximately 90% without the requirement of expensive, time consuming laborious purification steps to achieve this degree of purity. Surpassing the purity of commercial preparations comprising CRL thus illustrating the enormous contribution rendered to the art in a field full of unpredictable events as is evidenced by the prior art covering this subject.

The production of CRL by *Pichia* cells harbouring pPICpp*LIP* plasmid was confirmed by amino terminal sequencing of the protein concentrated from the culture supernatants. NH_2- amino-terminal sequence analysis indicated the presence of two species.

We also expressed the synthetic *C.rugosa* lipase in *S.cerevisiae* using a pYES2 plasmid in which the expression was under control of the *UAS-GAL* promoter, showing that the optimisation of the expression in another host than *P. pastoris* can be carried out and that the invention covers expression in any industrially applied host cell used for protein production and preferably also secretion. In particular yest cells.

(2) Characterisation of the recombinant lipase

The clone with the highest level of lipase secretion, selected from the transformants harbouring the plasmid pPICprepro*LIP* was employed for the production on a larger scale of the recombinant CRL (rCRL). 1 l culture in rich standard medium was maintained at pH 7.8 and followed for 100 h. The final lipolytic activity in the supernatant was 125 U/ml (Fig. 3a). To further improve the lipase expression level in the culture supernatant, we carried out a high density fermentation of the same clone, using the conditions reported by Payne et al [29]. Lipase activity accumulated to 150 U/ml after 100 h fermentation in 1 l culture (Fig. 3b), but the culture productivity remained in the same range of 450 U/l*h. This expression level can be improved by amendment of the fermentation conditions as will be apparent to a person skilled in the

art. No special conditions have been used in the examples for high protein production level. We just used methanol or galactose as inducers.

SDS-PAGE analysis of rCRL present in the culture supernatant showed a single protein band having a molecular weight of 60 kDa, (fig. 2), which corresponds to the molecular size reported for the native lipase isoforms [4,6].

The deduced amino acid sequence of rCRL contains three potential *N*-glycosylation sites [30] at position 291, 314 and 351. Hence, after deglycosylation a decrease in the molecular weight of ca. 3 kDa was observed, showing that the recombinant protein has a carbohydrate content of 5%. Lip1 was deglycosylated before and after denaturation; identical molecular weights were observed, but the deglycosylation reaction on the protein in native state required an almost double incubation time.

For native lipases A and B isolated after HPLC purification, carbohydrate contents between 3.6-8% have been described [6]. A slightly reduced carbohydrate content of 3-4 % was determined for native commercial CRL, by comparison of the two glycosylated forms.

To define the catalytic specificity of the expressed lipase, activity assays with substrates differing in chain length were carried out. We tested the commercial and the recombinant lipase for their ability to hydrolyse pure medium-chain triglycerides (MMM) and long-chain triglycerides (LLL). For both lipases, the highest activity was observed towards tricaprin (C10) rather than towards triolein, but a relatively high hydrolytic activity was found also on long-chain triglycerides when a natural oil, such as cocoa butter was used. Our data are also in good agreement with literature data concerning other commercial preparations of *C. rugosa* lipases [31] The recombinant and commercial CRL showed rather different specificity profiles when their activities were measured on acylglycerides with a wider range of chain-length. The assays were carried out on acylglycerides methyl esters (m.p. 20-54 °C) and revealed a maximum activity towards palmitate (C16) for the commercial CRL and towards caprinate (C10) for the recombinant one. Eventhough the commercial CRL shows a rather high relative activity on tricaprinate (C10), it seems to have a more marked preference for longer (C14-C16) acyl chains in comparison to the recombinant lipase (Fig. 4). We consider these characteristics due to the presence in the commercial preparation of, at least, one isoform with more pronounced or prominent preference for long chain acylglycerols. Thus, the commercial CRL substrate specificity profile can be interpretated as resulting from the

superimposition of different curves, each representing the fatty acid chain length profile of a single lipase isoform. As the commercial mixture also contains the *LIP1* gene product, the two substrate specificity profiles are in good agreement for short-middle (C4-C12) chain length, but diverge strongly for the longer (C14-C20) acyl chains.

After isoelectric focusing two bands with slightly different pI of 3.9 and 4.0 were seen on the gel possibly due to different glycosylation forms.

Since Rua *et al* [6], reported a pI between 4.8 and 5.04 for the four components of lipase B, whose N-terminal sequence coincides with the aa sequence deduced for LIP1, rCRL expressed in *Pichia pastoris* showed an isoelectric point slightly lower than expected.

The temperature and pH optimum for the recombinant and the commercial CRL were compared, as shown in Tab. II. The recombinant and commercial lipases were most active at 30°C-40°C. Maximum lipase activity was found at pH 7.0, while at pH 8.0 there was only 60% of the maximal activity

The fact that the physical-chemical, as well as the catalytic properties of rCRL in most cases coincide or, in general are in good agreement with those experimentally determined for the commercial preparation, represents further proof of the hypothesis that *LIP1* encodes the major isoform present in the commercial CRL preparation.

On the other hand, the presence of other isoforms in the commercial preparation, might explain the differences found in stability and optimal activity.

EXPERIMENTAL PROTOCOL

Materials

Restriction enzymes, DNA-modifying enzymes, T4-DNA ligase and Taq polymerase were from MBI Fermentas (St. Leon-Rot, Germany), Taq Dye Cycle Sequencing Kit was from Applied Biosystems (Weiterstadt, Germany), DNA Gel-Extraction Kit, Midi Plasmid Kit and Prepspin Plasmid Kit were from Qiagen (Hilden, Germany), lipase type VII from *C.rugosa*, different triacylglycerols were from Sigma (Deisenhofen, Germany), *Streptomyces plicatus* endo-β-N-acetylglucosaminidase H was from Boehringer Mannheim (Mannheim, Germany), pepton, yeast extract, yeast nitrogen base, malt extract were from Difco (Augsburg, Germany). All reagents were of analytical grade unless otherwise stated.

Strains, plasmids and media

E. coli DH5α (F⁻ endA1 hsdR17 (rk⁻, mk⁺) sup E44 thi1 λgyrA96 relA1 Δ(argF laczya) U169) was the host for plasmid amplification. *Pichia pastoris* GS115 (his4) (Invitrogen), *S cerevisiae* INVSC2 (Invitrogen) and X4004-3A (MATα, lys5, met2, ura3, trp1) were used for the expression of recombinant lipase.

Plasmid pUC19 was purchased from Pharmacia; PCYTEP1 (pT1) was obtained from the expression vector pT1-OmpAROL (25,26). pYES2 and pPICZαB were supplied from Invitrogen.

E. coli was grown at 37°C in Luria-Bertani medium (LB) containing 100 μg/ml ampicillin for selection of clones transformed with the vector pYES2 or 25 μg/ml zeocin for selection of clones transformed with the vector pPICZαC. *P. pastoris* was grown in shaking flasks at 30 °C, in a medium containing 1% yeast extract, 2% peptone, 100 mM potassium buffer pH 6.0, 1.34% yeast nitrogen base, $4*10^{-5}$% biotin, 1% glycerol (BMGY) before the induction, or 0.5% methanol (BMMY) for the induction.

Batch growth of *S.cerevisiae* was at 30°C in 0.67% w/v Yeast Nitrogen Base (Difco, UK) minimal medium supplemented with the appropriate amino acid at 50mg/l and either 2% glucose or 2% galactose as the carbon source.

For maintaining yeast cultures and plates YEPD medium was used (1% yeast extract, 2% peptone, 2% dextrose), and for selection of *P.pastoris* transformants YEPDS (YEPD + 2% sorbitol) plates containing zeocin (100 μg/ml) were used.

The procedure for culture of the transformed *S.cerevisiae* cells, protein extraction and detection have been described elsewhere [12].

Recombinant DNA techniques and DNA-sequencing

Standard recombinant DNA methods were carried out according to the methods described in Sambrock *et al.* [29] and Ausubel *et al.* [21]. Sequencing was performed by the fluorescence-based dideoxy DNA cycle sequencing method. The Taq Dye Deoxy™ Cycle Sequencing Kit and the 373A DNA Sequencing System were purchased by Applied Biosystems and used according to the manufacturer's instructions.

DNA-constructions

Gene synthesis

(1) Gene design

The gene was divided into 4 fragments of ca. 400 bp each, which

were separately cloned. The four resulting subconstructs were as follows: subassembly or cassette I (4 oligos, average length = 112mer), cassette II (6 oligos, average length = 92mer), cassette III (6 oligos, average length = 92mer), cassette IV (6 oligos, average length = 92mer)

In fig. 1 the lengths and the positions of oligonucleotides are indicated covering the 1688 b of the *LIP1* gene. Each pair of oligonucleotides included a 20 bp duplex segment to promote the annealing stability during the mutually primed synthesis. Additional residues were added beyond sites used for cloning at 5`- and 3`-terminus of the oligonucleotides, to allow efficient enzyme cleavage. In the case of cassette I and cassette IV, the synthesis of starting (5`-terminus in cassette I) and terminal (3`-terminus in cassette IV) extremities were extended beyond their normal end point to generate ends containing convenient cloning sites also present in pUC19 vector polylinker.

(2) Cassettes preparations

The four cassettes were synthesised in one step, by using the mutually priming long oligonucleotides in PCR. This procedure allowed the synthesis of ca. 400 bp fragments corresponding to cassette I to IV, that were cloned as blunt-end into pUC19 vector linearised at the *Sma*I site. The cassette sequences were checked by automated sequencing. Although the mutation rate observed was rather low (2/400 nt), it was necessary to carry out a repair strategy in order to remove sequence variants that appeared in the final cloned products. We employed PCR Site Directed Mutagenesis and current molecular biology techniques to remove undesired mutations. A number of restriction sites present in the synthetic gene and in the cloning vector pUC19 were used in order to combine in one copy error-free, different sequences coming from distinct clones of the same cassette. The four error-free subassemblies, cloned into pUC19 vector were designated as pUC-I to pUC-IV.

(3) Assembly of the final codon-optimised gene

The complete codon-optimised synthetic lipase gene was assembled into pUC19 vector. The *Xma*I/blunt-*Sal*I fragment from pUC-II was purified and ligated into pUC-III, which had been linearized by double digestion with *Pst*I/blunt and *Sal*I. The thus-created plasmid, containing the cassette II and the cassette III were joined at the *Sal*I site, and was designed as pUC-(II-III).

The *Xma*I-*Eco*RI fragment from pUC-(II-III) was purified and ligated into

pUC-I, which had been linearized by double digestion with *Xma*I and *Eco*RI. In this step, the cassettes I, II and III were joined into a plasmid that was called pUC-(I-III). Finally, the *Eco*RI-*Eco*RI fragment from pUC-IV was purified and ligated into pUC-(I-III), which had been linearised by digestion with *Eco*RI and dephosphorylated with CIP. The correctly assembled lipase cloning vector, pUC-(I-IV), was identified and characterised by restriction enzyme analysis, and the entire sequence was reconfirmed by automated sequencing.

Cloning of recombinant CRL in expression vectors

(1) Cloning of CRL genes into an expression vector for P.pastoris

The synthetic CRL gene was transferred into the pCyTexP1 vector as intermediate step for the in frame fusion of the gene with the *S.cerevisiae* α-factor leader sequence. The *Bam*HI fragment from pUC(I-IV) was ligated in *Bam*HI linearised and dephosphorylated pCytexP1 vector. The resulting plasmid was designated pCy*LIP*. In order to put the sequence encoding the mature lipase directly in frame with the α-factor signal sequence present in the *Pichia pastoris* expression vector pPICZαB, PCR was performed. The oligos used in the PCR were L1 (5'-CTG ACA GTT TAA ACG CTG TCT TGG-3'), complementary to the pPICZαB sequence including the *Pme*I site and L2 (5'-AGC TTC AGC CTC TCT TTT CTC TCC GAC TTC GAC GGG GTT GGC GGT GAA ACC GAT TGC-3'), complementary to the 3'end of the α-factor signal sequence and including the 5'sequence of the mature lipase along with a *Bgl*I site. The PCR product was directly cloned in pCytexP1 *Sph*I/blunt linearised giving the plasmids pCypreproafactor. The *Bgl*I fragment from pCy*LIP*, containing all the mature form of the lipase synthetic gene was ligated with the complementary *Bgl*I fragments from the plasmid pCypreproafactor. The efficiency of this cloning was improved by the fact that the ligations restored the Ampicillin resistance gene contained into the vector. The obtained plasmid was called pCypp*LIP*.

The plasmid pCypp*LIP* containing the gene for the mature synthetic lipase, in frame with prepro α-factor leader sequence was *Bam*HI/blunt-*Hind*III digested and the resulting fragment was ligated into pPICZαB linearised with *Xba*I/blunt-*Hind*III, giving pPICpp*LIP*.

(2) Cloning of CRL genes into expression vector for S.cerevisiae

As the expression vector in *S.cerevisiae* cells, we employed pYES2, a 2μ- based vector containing the *GAL1-GAL10* promoter, able to induce high level expression of genes cloned in its proximity upon growth in

galactose medium[21].

pPICnl*LIP* was *Bam*HI digested and ligated into pYES2 linearised with the same enzyme and dephosphorylated, giving pYnl*LIP*. pYnl*LIP* was used in turn for the cloning of the synthetic lipase gene preceded by prepro-α-factor leader sequences into vector pYES2. The *Hind*III-*Bste*II fragment containing the recombinant gene was isolated from pPICpp*LIP* and inserted in pYES2 digested with the same enzymes. The plasmid thus obtained was designated as pYpp*LIP*.

(3) Expression of C.rugosa *synthetic genes in* P.pastoris

Pichia pastoris GS115 cells were transformed with pPICnl*LIP*, pPICpp*LIP* and pPICpro*LIP*, by electroporation and transformations were plated onto solid selective medium (YEPD containing zeocin). Positive transformants selected on the basis of their ability to grow in the presence of zeocin, were checked for lipase activity. Colonies from initial transformation plates were picked out in replica onto minimal tributyrin-methanol plates. The tributyrin-methanol plates were incubated at 30 °C for 48 hrs with 0,1 ml of methanol being added to the lid of each plate every 24hrs. Direct evidence of lipase activity was provided using these plates, where the positive colonies appeared surrounded by a clear halo as compared to the opaque background of the medium, due to the tributyrin emulsion. All transformants obtained with pPICpp*LIP* displayed, to a different extent, lipase activity after over night incubation. A suitable negative control (*Pichia* cells transformed with pPICZαB) never formed halos, even after several days of incubation under inducing conditions.

(4) Expression of C.rugosa *synthetic genes in* S.cerevisiae

S.cerevisiae Invsc2 cells were transformed with pYpp*LIP* by electroporation. Transformations were plated onto solid minimal medium containing tributyrin and galactose. Transformants, selected on the basis of their ability to grow in the absence of leucine, were directly screened for lipase production and secretion, by being the positive colonies surrounded by a transparent halos as compared to the opaque background of the medium containing tributyrin emulsion.

Fermentations

The fermentation was carried out in a 1 l bioreactor (Braun) at 30 °C, in a rich standard medium at pH 6.0 containing 1% yeast extract, 2% peptone, 1.34% yeast nitrogen base, $4*10^{-5}$ % biotin and 0.5 % methanol.

For the high density fermentation, a complex medium containing 0.3% yeast extract, 0.5% pepton, 0.5% malt extract, FM21 (3.8 g KH_2PO_4, 0.97 g $(NH_4)_2SO_4$, 1.1 g $MgSO_4*7\ H_2O$, 0.08 g $CaSO_4*2H_2O$ in 1l), $1.3*10^{-3}$% PTM1 (6.0 $CuSO_4*5\ H_2O$, 0.8 g KI, 3.0 g $MnSO_4*\ H_2O$, 0.2 g $NaMoO_4*2H_2O$, 0.03 g H_3BO_3, 0.5 g $CoCl*6\ H_2O$, 20.0 g $ZnSO_4$, 65.0 g $FeSO_4*7\ H_2O$, for 1l), $2*10^{-5}$% biotin was employed at pH 7.5. The bioreactors were inoculated with 50 ml of a flask culture grown overnight to an OD_{600} of 2-3 in BGMY medium. The culture broth was maintained at constant pH by adding 2 M HCl and 2 M NaOH. The stirring rate was 350 rpm and the aeration rate was 1 l/min. The lipolytic activity of the supernatants and the cell wet weight were monitored throughout the fermentation.

5 ml of methanol were added daily to the bioreactor. In the case of high density fermentation, methanol was added after the cell culture reached 75 mg/ml of wet weight.

Enzyme assay

The fermentor broth was clarified by centrifugation at 30.000 g for 20 min at 4°C. Sodium azide was added to the fermentor broth at a final concentration of 0.03% w/v as bacteriostatic agent, before adjusting to pH 7.5 with sodium hydroxide. All the measurements for the physical-chemical, as well as for the catalytic characterisation, were carried out directly on the clarified fermentor broth, without any kind of further purification.

Lipase activity was routinely measured by a pH-stat. 66 mM tributyrin were emulsified in distilled water containing gum arabic (20 mg/ml) as stabiliser using a homogeniser (Ultraturrax T25, Janke & Kunkel) for 7 min at maximum speed. 20 ml of the substrate solution were heated to 30°C and adjusted to pH 7.2. After the addition of 10-200 μl of the enzyme solution, the activity was measured with a pH-stat (Metrohm). One unit was defined as the amount of enzyme which released 1 μmol fatty acid per minute.

For the determination of substrate specificity, 20 mM of triacylglycerols, and 5% w/v of cocoa butter and 100 mM of fatty acid methyl esters were each emulsified in distilled water, containing gum arabic (20 mg/ml), and used as substrate solution in the pH-stat assay.

The tristearin emulsion was obtained adding 5% v/v aceton, being

verified that, under the same conditions the lipases retained 47% of their full activity on tributyrin.

N-terminal sequencing

Amino terminal sequence analysis was performed on the recombinant lipases produced during the cultivation in flasks. The cells were separated by centrifugation and the resulting supernatants (180 ml) were ultrafiltered through a 50 kDa membrane. The resulting lipase solutions had a final volume of 20 ml and a concentration of 800 U/ml for ppLIP and preLIP. For the protein sequencing a gas-phase sequencer 470A (Applied Biosystems)was used following the manufacturer's instructions. After blotting, the PVDF membranes were stained with Coomassie Brilliant Blue R-250, and the lipase bands cut out and used for N-terminal sequence determination.

Protein determination

Protein concentration was determined with the bicinchoninic acid (BCA) protein assay kit (Pierce) using the enhanced method according to the manufacturer's instructions (Pierce, Instructions 23220/23225) and bovine serum albumin as standard.

Endo-β-N-acetylglycosaminidase H digestion

Protein samples were incubated for 12 h with endo-β-N-acetylglucosaminidase H (25 mU/mg protein) at 37 °C in a 50 mM potassium acetate buffer, pH 5.5, containing 0.5 mM phenylmethylsulphonyl fluoride to prevent proteolysis. For the deglycosylation of denatured samples, the protein was first incubated with 0.01% (m/v) SDS at 95°C for 3 min.

Isoelectric focusing and SDS-polyacrylamide electrophoresis

Analytic SDS-PAGE (8-25%) and IEF (pH 3-9) were performed with a Pharmacia Phast System according to the manufacturer's recommendations. The gels were stained for protein detection by a silver staining procedure [32]. Preparative gel electrophoresis was carried out in a 12.5% polyacrylamide gel according to Laemmli [33] and proteins were stained with Coomassie Brilliant Blue R-250.

Effect of temperature and pH on enzyme stability and activity

The effect of pH on the enzyme activity was determined by pH-stat assay at 30°C using tributyrin as substrate. The effect of pH on lipase

stability was determined by incubating aliquots of lipase solution for 20 h at 4°C in 0.1 M phosphate buffers at different pHs. Residual activity was measured by pH-stat assay. The optimum temperature for enzyme activity was determined at pH 7.2 and various temperatures with tributyrin as substrate. The effect of temperature on lipase stability was determined by incubating aliquots of lipase solution for 30 min in 25 mM Tris-buffer, pH 7.5 at various temperatures. Residual activity was measured by pH-stat assay. The heat-inactivation curves were determined measuring the residual activity after a different incubation time at 50°C in 25 mM Tris-buffer, pH 7.5

Tab. I: Characteristics of mutant *Candida rugosa* genes produced by site-directed mutagenesis

Tab. II: Comparison of properties of commercial and recombinant lipase from *Candida rugosa*.
Abbreviations: CRL, *Candida rugosa* lipase; N.D., not determined; MW, molecular weight; T_{opt}, temperature optimum of activity; T_{stab}, temperature stability after 30 min of incubation; pH_{opt}, pH optimum of activity; pH_{stab}, pH stability ; pNPP, p-nitrophenyl palmitate.

TABLE I

CHARACTERISTICS OF MUTANT *Candida rugosa* LIPASE GENES PRODUCED BY SITE-DIRECTED MUTAGENESIS

Mutant	Number of mutated CUG Ser residues	Position of mutated CUG Ser residues
1	2	Ser^{209}, Ser^{241}
2	3	Ser^{209}, Ser^{241}, Ser^{282}
3	5	Ser^{209}, Ser^{241}, Ser^{282}, Ser^{348}, Ser^{349}
4	8	Ser^{209},Ser^{241},Ser^{282},Ser^{348},Ser^{349},Ser^{365},Ser^{436}, Ser^{440}

TABLE II

COMPARISON OF PROPERTIES OF COMMERCIAL AND RECOMBINANT LIPASES FROM *Candida rugosa*

Properties	Native CRL	Recombinant CRL
MW (Da)	60,000	60,000
pI	N.D.	3.9–4.0
T_{opt}(°C)	30–40	30–40
pH_{opt}	6.5–7.5	6.5–7.5
T_{stab} (°C)	50	50
pH_{stab}	8.0–8.5	8.0–8.5

Abbreviations: CRL, *Candida rugosa* lipase; N.D., not determined; MW, molecular weight;

T_{opt}, temperature optimum of activity; T_{stab}, temperature stability after 30min of incubation;

pH_{opt}, pH optimum of activity; pH_{stab}, pH stability ;

Figure legends:

Fig. 1: Design of the synthetic gene. Positions and lengths of oligonucleotides covering the Lip 1 gene (1647 bp) are indicated by arrow. Each cassette was synthesised using 2 or 3 couples of long overlapping oligonucleotides, represented as solid bars. Separately synthesised gene cassettes were cloned into pUC19 vector and ligated in order to assemble the entire gene.

Fig. 2: SDS-PAGE analysis of recombinant *Candida rugosa* lipase. Lane 1: molecular weight standard in kDa; lane 2: lipase from 0,5 µl culture supernatant was withdrawn after 5 days induction; lane 3: 0,5 µl of supernatant as withdrawn from 5 days non-induced culture.

Fig. 3: Recombinant lipase production during cultivation with different media. Fermentative production of lip1 in a 1l bioreactor using (A) standard conditions: BMMY medium, 30°C and pH 6.0 and (B) high cell density conditions are reported by Payne et al. at 30°C and pH 6.0. The lipase activity was measured with a pH-stat, using tributyrin as substrate, at 30°C and pH 7.2. The lipase activity was measured with a pH-stat, using tributyrin as substrate, at 30 °C and pH 7.2.

Fig 4: Substrate specificity of recombinant and commercial *C.rugosa* lipase. Activity towards various triacylglycerides (A) and fatty acid methyl esters (B) of different chain length of the acyl group (C2 to C22). Cocoa butter (C16-18) contains predominantely palmitic acid (C16) in the sn-1, oleic acid (C18:1) in the sn-2 and stearic acid (C18) or palmitic acid (C16) in the sn-3 position of the tracylglycerool. Relative activities were determined by pH-stat assay at pH 7,2 and 30°C for triacylglycerols and 50°C for fatty acid methyl esters.

Sequence Listing: DNA-sequence of synthetic and native lipase I.
The original sequence has no 985229. Below this sequence the synthetic gene of the examples is illustrated. The original sequence corresponds to the sequence id no 1 giving a nucleotide sequence for native lipase 1 and the corresponding amino acid sequence upon translation of said sequence. The synthetic gene is sequence id. no. 2.

Align Results

No. 985229	original sequence
unmarked	modified sequence of the synthetic gene

```
>_                                                          1647 nt vs.
>_                                                          1647 nt
scoring matrix: , gap penalties: -12/-2

77.1% identity;          Global alignment score: 3945

                10        20        30        40        50
60
985229
ATGGAGCTCGCTCTTGCGCTCAGCCTCATTGCCTCGGTGGCTGCTGCCCCCACCGCCACG
       :::::  : ::: : ::  :     : :::::::: :: ::::::::::: :
::::::::

ATGGAATTGGCTTTGGCTTTGTCTTTGATTGCCTCCGTTGCTGCTGCCCCAACCGCCACT
                10        20        30        40        50
60

                70        80        90       100       110
120
985229
CTCGCCAACGGCGACACCATCACCGGTCTCAACGCCATCATCAACGAGGCGTTCCTCGGC
       : :: ::::: :::::::::::::::: : :::::::::::::::: :: ::: :
::

TTGGCTAACGGTGACACCATCACCGGTTTGAACGCCATCATCAACGAAGCCTTCTTGGGT
                70        80        90       100       110
120

               130       140       150       160       170
180
985229
ATTCCCTTTGCCGAGCCGCCGGTGGGCAACCTCCGCTTCAAGGACCCCGTGCCGTACTCC
       ::::: :::::::: :: :: :: :: ::: :  : :::::::::: :: ::
::::::

ATTCCATTTGCCGAACCACCAGTTGGTAACTTGAGATTCAAGGACCCAGTTCCATACTCC
               130       140       150       160       170
180

               190       200       210       220       230
240
985229
GGCTCGCTCGATGGCCAGAAGTTCACGAGCTACGGCCCGAGCTGTATGCAGCAGAACCCC
       :: ::  : ::::: :: ::::::::    ::::: ::    :::::::: ::
:::::

GGTTCCTTGGATGGTCAAAAGTTCACTTCTTACGGTCCATCTTGTATGCAACAAAACCCA
               190       200       210       220       230
240

               250       260       270       280       290
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300
985229
GAGGGCACCTACGAGGAGAACCTCCCCAAGGCAGCGCTCGACTTGGTGATGCAGTCCAAG
      :: :: :::::::: :: ::: : :: ::::::::  : ::  :::: :::::
:::::

GAAGGTACCTACGAAGAAAACTTGCCAAAGGCAGCTTTAGATCTGGTTATGCAATCCAAA
               250       260       270       280       290
300

               310       320       330       340       350
360
985229
GTGTTTGAGGCGGTGAGCCCGAGCAGCXGAGGACTGTCTCACCATCAACGTGGTGCGGCC
       :: :: :: :: ::   : : : :  :.:: :::::: : ::::: :: :: ::
: ::
       GTTTTCGAAGCTGTTT-
CTCCATCTTCTGAAGACTGTTTGACCATTAATGTTGTTAGACC
               310       320       330       340       350

               370       380       390       400       410
420
985229
GCCGGGCACCAAGGCGGGTGCCAACCTCCCGGTGATGCTCTGGATCTTTGGCGGCGGGTT
        :: :: :: ::::: ::::::::: : :: :: ::: : :::::::::: ::
:: ::

ACCCGGGACAAAGGCTGGTGCCAACTTGCCAGTTATGTTGTGGATCTTTGGTGGTGGTTT
     360       370       380       390       400       410

               430       440       450       460       470
480
985229
TGAGGTGGGTGGCACCAGCACCTTCCCTCCCGCCCAGATGATCACCAAGAGCATTGCGAT
       ::: :: ::::: :: :: :::::::::: ::::: ::::: ::::::
::::: ::

TGAAGTTGGTGGTACTAGTACCTTCCCTCCAGCCCAAATGATTACCAAGTCTATTGCTAT
     420       430       440       450       460       470

               490       500       510       520       530
540
985229
GGGCAAGCCCATCATCCACGTGAGCGTCAACTACCGCGTGTCGTCGTGGGGGTTCTTGGC
       ::: ::::: ::::::::::    ::::::::: : :: :::   :::::
::::::::

GGGTAAGCCAATCATCCACGTTTCTGTCAACTACAGAGTCTCGAGCTGGGGTTTCTTGGC
     480       490       500       510       520       530

               550       560       570       580       590
600
985229
TGGCGACGAGATCAAGGCCGAGGGCAGTGCCAACGCCGGTTTGAAGGACCAGCGCTTGGG
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        ::: ::::: :::::::::: ::    ::::::::::::::::::::::::  :
:::::

TGGTGACGAAATCAAGGCCGAAGGTTCTGCCAACGCCGGTTTGAAGGACCAAAGATTGGG
      540        550        560        570        580        590

                610        620        630        640        650
660
985229
TATGCAGTGGGTGGCGGACAACATTGCGGCGTTTGGCGGCGACCCGACCAAGGTGACGAT
       :::::: :::::::: :::::::::: :: ::::: :: :: :: :: :::::
:: ::

TATGCAATGGGTGGCTGACAACATTGCTGCTTTTGGTGGTGATCCAACTAAGGTTACTAT
      600        610        620        630        640        650

                670        680        690        700        710
720
985229
CTTTGGCGAGAGCGCGGGCAGCATGTCGGTCATGTGCCACATTCTCTGGAACGACGGCGA
       :::::: ::    :: ::    ::::: :::::::: :::::: :
:::::::::: ::

CTTTGGTGAATCTGCTGGTTCTATGTCCGTCATGTGTCACATTTTGTGGAACGACGGTGA
      660        670        680        690        700        710

                730        740        750        760        770
780
985229
CAACACGTACAAGGGCAAGCCGCTCTTCCGCGCGGGCATCATGCAGAGCGGGGCGATGGT
       :::::: :::::::: :::::  : ::: : :: :: ::::::::    :: ::
:::::

CAACACTTACAAGGGTAAGCCATTGTTCAGAGCTGGTATCATGCAATCTGGTGCTATGGT
      720        730        740        750        760        770

                790        800        810        820        830
840
985229
GCCGAGCGACGCCGTGGACGGCATCTACGGCAACGAGATTTTTGACCTCTTGGCGTCGAA
        ::    :::::::: ::::: :::::::: ::::: ::::::::: : :::::
:: ::

TCCATCTGACGCCGTCGACGGTATCTACGGTAACGAAATTTTTGACTTGTTGGCTTCCAA
      780        790        800        810        820        830

                850        860        870        880        890
900
985229
CGCGGGCTGCGGCAGCGCCAGCGACAAGCTCGCGTGCTTGCGCGGTGTGAGCAGCGACAC
       ::: :: :: ::    :::   :::::: : :: :: ::: : :::::
:::::
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CGCTGGTTGTGGTTCTGCCTCTGACAAGTTGGCTTGTTTGAGAGGTGTTTCTTCTGACAC
      840        850        860        870        880        890

                910        920        930        940        950
960
985229
GTTGGAGGACGCCACCAACAACACCCCTGGGTTCTTGGCGTACTCCTCGTTGCGGTTGAG
         ::::: :::::::::::::::::::::: :::::::: :::::::: ::   :
:::

TTTGGAAGACGCCACCAACAACACCCCTGGTTTCTTGGCTTACTCCTCCTTAAGATTGTC
      900        910        920        930        940        950

                970        980        990       1000       1010
1020
985229
CTACCTCCCCCGGCCCGACGGCGTGAACATCACCGACGACATGTACGCCTTGGTGCGCGA
         ::: : ::  : :: ::::: :: :::::::::::::::::::::::: :::::
: ::

TTACTTGCCAAGACCAGACGGTGTTAACATCACCGACGACATGTACGCTTTGGTTAGAGA
      960        970        980        990       1000       1010

               1030       1040       1050       1060       1070
1080
985229
GGGCAAGTATGCCAACATCCCTGTGATCATCGGCGACCAGAACGACGAGGGCACCTTCTT
         :: :::::::::::::::::::: :::::::: ::::: :::::::: ::
::::::::

AGGTAAGTATGCCAACATCCCTGTTATCATCGGTGACCAAAACGACGAAGGTACCTTCTT
     1020       1030       1040       1050       1060       1070

               1090       1100       1110       1120       1130
1140
985229
TGGCACCAGCAGCTTGAACGTGACCACGGATGCCCAGGCCCGCGAGTATTTCAAGCAGAG
        ::: ::        :::::::: ::::: :::::::: ::: : ::
::::::::::

TGGTACTTCTTCTTTGAACGTTACCACTGATGCCCAAGCCAGAGAATATTTCAAGCAATC
     1080       1090       1100       1110       1120       1130

               1150       1160       1170       1180       1190
1200
985229
CTTTGTCCACGCCAGCGACGCGGAGATCGACACGTTGATGACGGCGTACCCCGGCGACAT
         :::::::::: :::::::: :: :::::::: :::::::: :: ::::: ::
:::::

TTTTGTCCACGCTAGCGACGCTGAAATCGACACTTTGATGACTGCTTACCCAGGTGACAT
     1140       1150       1160       1170       1180       1190
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             1210       1220       1230       1240       1250
1260
985229
CACCCAGGGCAGCCCGTTCGACACGGGTATTCTCAACGCCCTCACCCCGCAGTTCAAGAG
       ::: :: ::    :: :: ::::: :: ::::: :::::: : ::::: ::
::::::::

CACTCAAGGTTCTCCATTTGACACTGGAATTCTAAACGCCTTGACCCCACAATTCAAGAG
    1200       1210       1220       1230       1240       1250


             1270       1280       1290       1300       1310
1320
985229
AATCAGCGCGGTGCTCGGCGACCTTGGCTTTACGCTTGCTCGTCGCTACTTCCTCAACCA
       ::::   :: ::  : :: ::: : :: :::::  : :::::: : :::::: :
:::::

AATCTCTGCTGTTTTGGGTGACTTGGGTTTTACTTTGGCTCGTAGATACTTCTTGAACCA
    1260       1270       1280       1290       1300       1310


             1330       1340       1350       1360       1370
1380
985229
CTACACCGGCGGCACCAAGTACAGCTTCCTCAGCAAGCAGCTCAGCGGCTTGCCGGTGCT
       ::::::::: :: :::::::::   ::: :    ::::  :    :: :::::
::  :

CTACACCGGTGGTACCAAGTACTCTTTCTTGTCTAAGCAATTGTCTGGTTTGCCAGTTTT
    1320       1330       1340       1350       1360       1370


             1390       1400       1410       1420       1430
1440
985229
CGGAACGTTCCACTCCAACGACATTGTCTTCCAGGACTACTTGTTGGGCAGCGGCTCGCT
       :: :: :::::::::::::: :: :::::::: ::::::::::::::    ::
::  :

GGGTACTTTCCACTCCAACGATATCGTCTTCCAAGACTACTTGTTGGGTTCTGGTTCCTT
    1380       1390       1400       1410       1420       1430


             1450       1460       1470       1480       1490
1500
985229
CATCTACAACAACGCGTTCATTGCGTTTGCCACGGACTTGGACCCCAACACCGCCGGGTT
       :::::::::::::: :::::::: :::::::: ::::::::::: ::

GATCTACAACAACGCTTTCATTGCTTTTGCCACTGACTTGGACCCAAACACCGCCGGTTT
    1440       1450       1460       1470       1480       1490


             1510       1520       1530       1540       1550
1560
985229
```

```
GTTGGTGAAGTGGCCCGAGTACACCAGCAGCAGCCAGAGCXGGCAACAACTTGATGATGA
       :::::: :::::::: :: ::::::  :  :  :   : :.::
:::::::::::::::
_       GTTGGTTAAGTGGCCAGAATACACCT-
CTTCTTCTCAATCTGGTAACAACTTGATGATGA
    1500       1510       1520       1530       1540       1550


             1570       1580       1590       1600       1610
1620
985229
TCAACGCCTTGGGCTTGTACACCGGCAAGGACAACTTCCGCACCGCCGGCTACGACGCGT
       ::::::: ::::: :::::::::::: :::::::::::: : ::::::::
:::::::: :

TCAACGCTTTGGGTTTGTACACCGGTAAGGACAACTTCAGAACCGCCGGTTACGACGCTT
     1560       1570       1580       1590       1600       1610


             1630       1640
985229 TGTTCTCCAACCCGCCGAGCTTCTTTGTG
       :::::::::::: ::    ::::::::
_      TGTTCTCCAACCCACCATCTTTCTTTGTT
     1620       1630       1640

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CLAIMS

1. Nucleic acid sequence encoding a ripening form of native *C. rugosa* lipase, said ripening form being selected from pre, pro, prepro or mature lipase, said nucleic acid sequence comprising 60% or less of the CTG codons at positions encoding serine as present in the corresponding native *C. rugosa* encoding sequence, said CTG codons having been replaced by a universal codon for Serine, said lipase preferably being the lipase 1.

2. Nucleic acid sequence according to claim 1 wherein between 8 and 20 CTG codons encoding serine have been replaced by a universal codon for serine.

3. Nucleic acid sequence according to claim 1 wherein more than 70%, preferably more than 80%, most preferably more than 90% of the CTG codons at serine encoding positions have been replaced.

4. Nucleic acid sequence according to any of the preceding claims wherein all CTG codons at serine encoding positions have been replaced.

5. Nucleic acid sequence according to any of the preceding claims wherein the C and G nucleotide level has been reduced below 63% and wherein the amino acid identity remains that of the native lipase.

6. Nucleic acid sequence according to any of the preceding claims wherein a *Hin*dIII site in the coding region of the corresponding native gene has been amended thereby removing the restriction site yet maintaining the coding identity.

7. Nucleic acid sequence according to any of the preceding claims wherein the 5' terminus of the encoding sequence for the mature lipase is preceded by a leader sequence heterologous to *C. rugosa*.

8. Nucleic acid sequence according to claim 7 wherein the leader sequence is native to a yeast cell.

9. Nucleic acid sequence according to claim 7 or 8, wherein the leader sequence is a leader sequence derived from *S. cerevisiae*.

10. Nucleic acid sequence according to any of the preceding claims comprising one or more restriction sites absent in the native encoding sequence yet such that the amino acid sequence is unchanged at said location, wherein multiple restriction sites will be different such that each restriction site is unique to the sequence.

11. Nucleic acid sequence according to any of the preceding claims comprising additional nucleic acid sequences at the 5' and the 3' termini, these additional sequences comprising unique restriction sites for the sequence thereby enabling incorporation of the sequence encoding the lipase in an expression vector.

12. Nucleic acid sequence encoding a ripening form of native *C. rugosa* lipase, said ripening form being selected from pre, pro, prepro or mature, said nucleic acid sequence being a variant of a nucleic acid sequence according to any of the preceding claims, said variant comprising 60% or less of the CTG codons at positions encoding serine as present in the corresponding native *C. rugosa* encoding sequence, said CTG codons having been replaced by a universal codon for Serine, said lipase preferably being the lipase 1, said ripening form having the specificity of the corresponding native lipase.

13. A nucleic acid sequence according to claim 12, said ripening form being capable of hybridising under stringent conditions to the coding sequence of sequence id no 1.

14. A nucleic acid sequence according to claim 12 or 13, said nucleic acid sequence encoding a ripening form of lipase with an amino acid sequence exhibiting more than 88% identity with the corresponding native sequence, said native sequence e.g. being that of lipase I as encoded in sequence id. no. 1.

15. Expression vector comprising a nucleic acid sequence according to the previous claims operatively linked to a promoter.

16. Microorganism other than *C. rugosa* comprising a sequence or expression vector according to any of the preceding claims, preferably comprising more than one said sequence.

17. Microorganism according to claim 16 wherein the codons of the encoding nucleic acid sequence have been amended to correspond to codon usage of the microorganism.

18. Microorganism according to claim 16 or 17 being capable of secreting protein.

19. Microorganism according to any of claims 16-18 being *S. cerevisiae*.

20. Microorganism according to any of claims 16-18 being *P. pastoris*.

21. Microorganism according to any of claims 16-18 being *Hansenula*.

22. Microorganism according to any of the claims 16-21 capable of secreting said lipase to a degree such that said lipase is contaminated at the most by 20% of other *C. rugosa* protein in the supernatant of a culture of said microorganism, preferably at the most 10% more preferably at the most 5%.

23. *C. rugosa* lipase contaminated at the most by 20% of other *C. rugosa* protein, preferably at the most 10% more preferably at the most 5%.

24. *C. rugosa* lipase free of other *C. rugosa* lipase, i.e. homogenous *C. rugosa* lipase.

25. *C. rugosa* lipase according to claim 23 or 24, wherein the lipase is lipase 1 free of lipases 2-5 where lipase 1 is defined by the amino acid sequence of sequence id no 1 and/or an amino acid sequence exhibiting more than 88% amino acid identity of sequence id. no. 1.

26. *C. rugosa* lipase according to claim 25, wherein the lipase exhibits higher activity towards caprinate (C10) than towards palmitate (C16) as can be determined in pH stat assay at 30°C and pH 7.2.

27. *C. rugosa* lipase according to claim 25 or 26, wherein the lipase exhibits higher activity towards smaller acyl chains than for longer (C14-C16) acyl chains as can be determined in pH stat assay at 30°C and pH 7.2.

28. *C. rugosa* lipase according to any of claims 25-27 with a pI of 3.9 or 4.0.

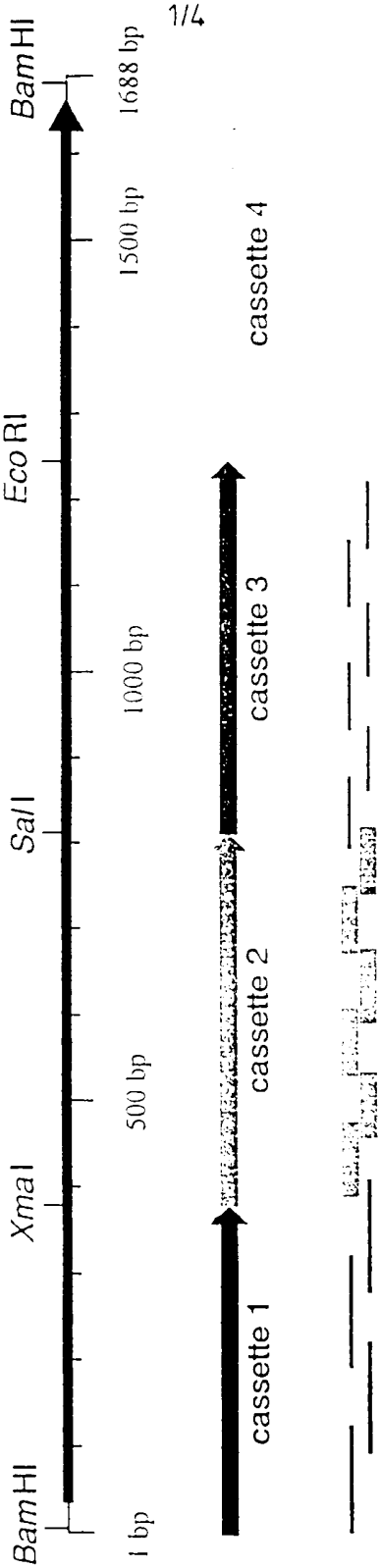
29. *C. rugosa* lipase according to any of claims 25-28 with a pH optimum of 7.0 and temperature for activity between 30 and 40C.

30. *C. rugosa* lipase according to any of claims 25-29 that is N glycosylated.

31. A process for industrial scale production of a lipase according to any of claims 25-30 comprising cultivation of a microorganism according to any of claims 16-22, isolation of the resulting expression product as active lipase in a manner known per se for microorganism protein production processes, said lipase preferably being present in the culture supernatant in an amount comprising over 80% of the total protein present.

32. Use of a lipase according to any of claims 25-30 in a manner known per se for lipase in a process requiring high specificity towards acyl chains shorter than C14, preferably on an industrial scale.

fig-1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XmaI
SalI
EcoRI
BamHI
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500 bp
1000 bp
1500 bp
1688 bp
cassette 1
cassette 2
cassette 3
cassette 4

Fig-2

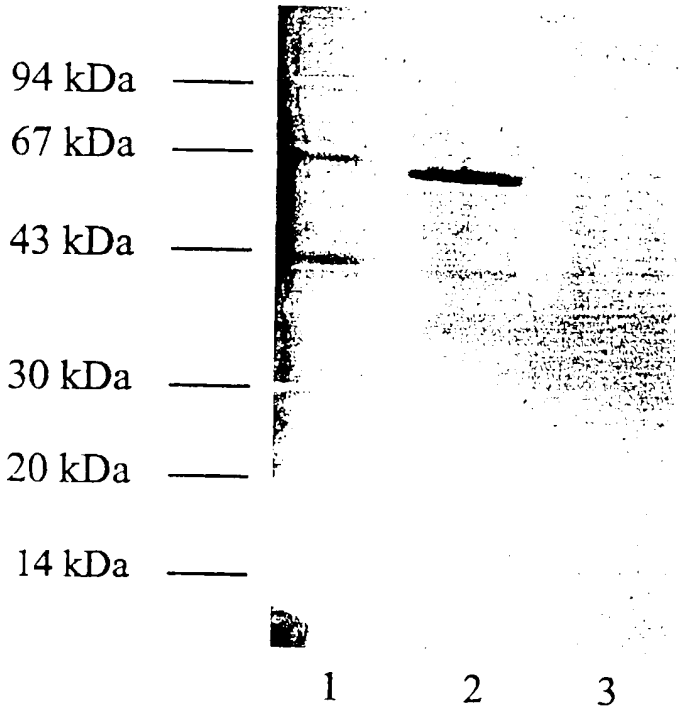
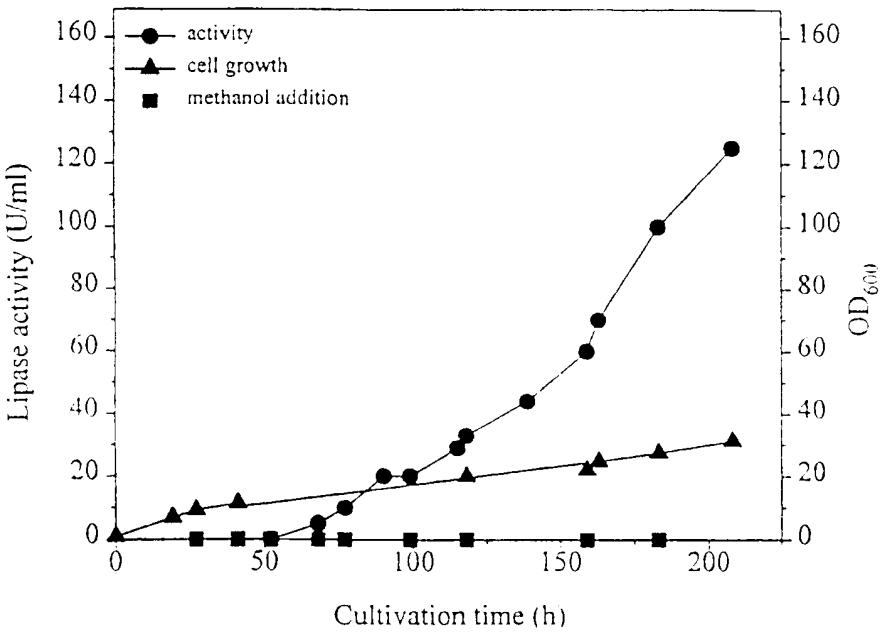


Fig-3

A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cell growth
methanol addition
Lipase activity (U/ml)
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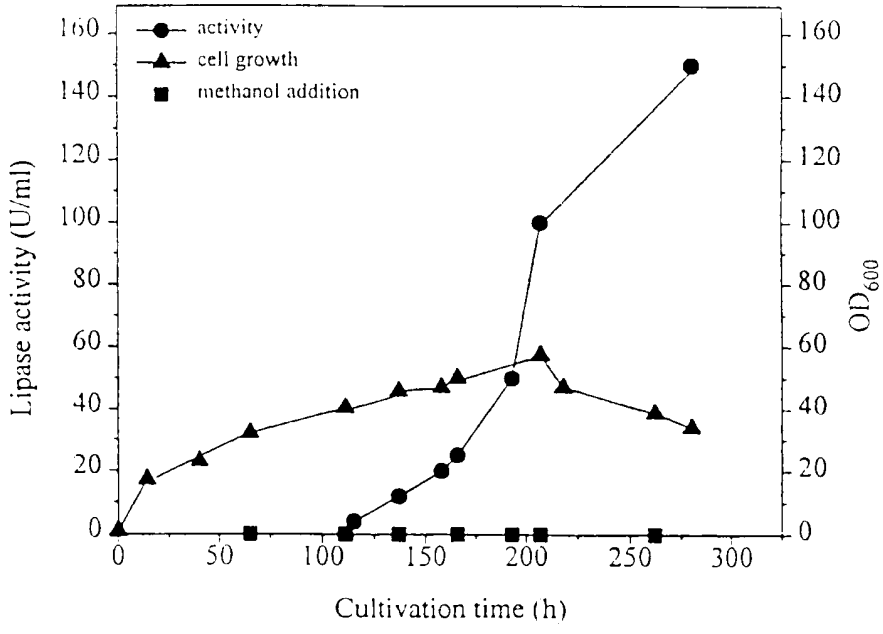
40
60
80
100
120
140
160
0
50
100
150
200

B


activity
cell growth
methanol addition
Lipase activity (U/ml)
OD600
Cultivation time (h)
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20
40
60
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100
120
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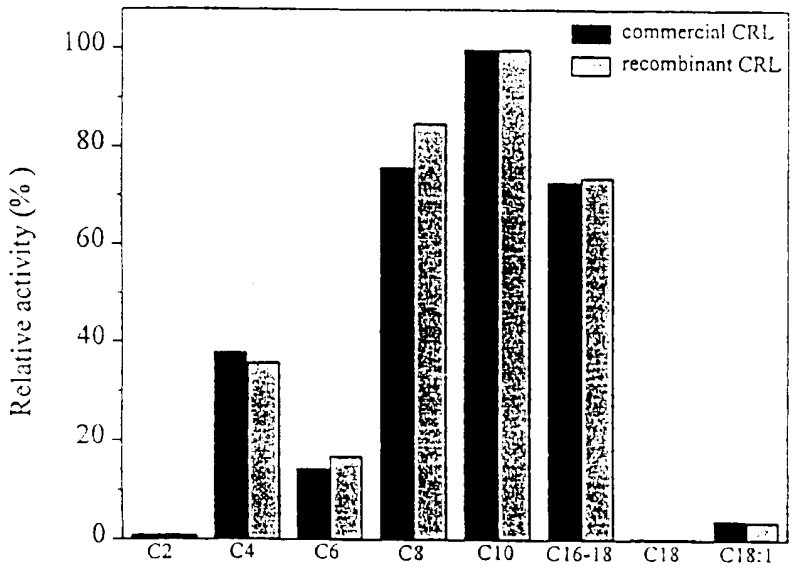
50
100
150
200
250
300

Fig-4

A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recombinant CRL
Relative activity (%)
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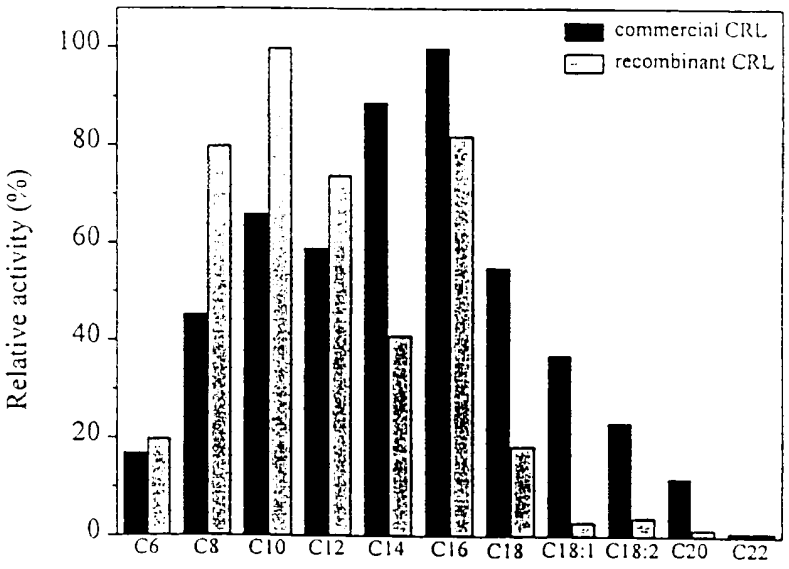
80
60
40
20
0
C2
C4
C6
C8
C10
C16-18
C18
C18:1

B


commercial CRL
recombinant CRL
Relative activity (%)
100
80
60
40
20
0
C6
C8
C10
C12
C14
C16
C18
C18:1
C18:2
C20
C22

INTERNATIONAL SEARCH REPORT

arnational Application No
PCT/NL 97/00524

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/55 C12N1/19 C12N9/20

According to International Patent Classification(IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
X	L. ALBERGHINA and M. LOTTI: "Protein engineering of a fungal Lipase" in ENGINEERING OF/WITH LIPASES, F. Malcata (ED.) NATO ASI Series E:Applied Sciences-Vol. 317 pages219-223 (1996). XP002067509 ---	1-22
X	F. FUSETTI ET AL.: "Effect of the leader sequence on the expression of recombinant C. rugosa lipase by S. cerevisiae cells" BIOTECHNOLOGY LETTERS, vol. 18, no. 3, March 1996, ISSN 0141-5492, pages 281-286, XP002067441 cited in the application see the whole article, especially the last paragraph on page 286 --- -/--	1-22

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
9 June 1998	23/06/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Cupido, M

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INTERNATIONAL SEARCH REPORT

ernational Application No
PCT/NL 97/00524

C (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication where appropriate, of the relevant passages	Relevant to claim No.
X	P. GROCHULSKI ET AL.: "Insights into interfacial activation from an open structure of Candida rugosa lipase" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 268, no. 17, 15 June 1993, MD US, pages 12843-12847, XP002067442 see page 12843, right-hand column, paragrapn 3 ---	23-30
X	WO 94 01542 A (CONSEJO SUPERIOR INVESTIGACIONES CIENTIFICAS) 20 January 1994 see examples 1-4 ---	31,32
A	M. LOTTI ET AL: "Variability within the Candida rugosa lipases family" PROTEIN ENGINEERING, vol. 7, no. 4, 1 April 1994, pages 531-535, XP000441770 cited in the application ---	1-22
T	L. ALBERGHINA & M. LOTTI: "Cloning, sequencing and expression of Candida rugosa lipase" METHODS IN ENZYNOLOGY, vol. 284, no. Lipases part A Biotechnology, 1997, pages 246-260, XP002067443 see page 257 - page 260 -----	1-22

2

INTERNATIONAL SEARCH REPORT

information on patent family members

ernational Application No
PCT/NL 97/00524

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9401542 A	20-01-1994	ES 2050068 A	01-05-1994